A METHOD FOR IDENTIFYING DNA MUTATION USING MICROWELLS AND KIT THEREFOR

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BACKGROUND OF THE INVENTION

Field of the Invention

The present invention relates to a method for identifying DNA mutation using microwells and a kit therefor, more particularly, to an economical, simple and safe method for identifying single base mutations including substitution, insertion and deletion using microwells, and a kit therefor.

Backgound of the Invention

Techniques for determining the species of organisms and diagnosing diseases by identification of genes have been widely used in the art(see: J. Clin. Microbiol., 34:130-133, 1996). Diseases can be treated by using a variety of medications depending upon species and types of diseases. However, in such cases where the diseases are attributable to the infection with microorganisms which can be mutated, the use of conventional medication is limited.

Conventional techniques for detection of small mutations such as substitution, insertion and deletion in a DNA sequence includes dot blotting, RFLP(restriction fragment-length polymorphism) analysis, SSCP(single strand conformation polymorphism) analysis and DNA sequencing:

First, dot blotting technique employs the principle of Southern blotting, in which even a single base pair mismatch causes disruption of a DNA duplex above a certain temperature. This dot blotting can determine the presence of mutations through detection of signals created by a target sequence-represented oligonucleotide probe which has been prelabeled with a radioisotope, a fluorescent dye or

an enzyme connected to a chromogenic response when the oligonucleotide probe hybridizes nucleic acids immobilized on the membrane(see: British J. Dermatol., 131:72-77, 1994). Also, through the immobilization of an oligonucleotide probe on the membrane and preamplification of a target DNA, a mutation can be determined by the duplex fomation between the immobilized oligonucleotide and the amplified DNA.

Secondly, RFLP analysis employs the characteristic of restriction enzymes which cleave only specific sequences. In other words, a normal nucleotide sequence amplified by PCR can be cleaved with a restriction enzyme while the corresponding nucleotide sequence with mutations on the recognition site cannot be cleaved by the same enzyme. After treating normal and mutated DNA samples with a restriction enzyme, the resulting mixtures of DNA fragments are subject to electrophoresis side by side on the same gel, and the presence of mutation can be determined by comparing the number of DNA fragments from each sample(see: Mol. Cell Biol., 279-281, 1995).

Thirdly, SSCP analysis employs the characteristic of single-stranded DNA in which its conformational changes caused by even a single point mutation give rise to changes in migration of the fragments in a non-denaturing gel. When the pattern of the restriction cleavage of a normal DNA and that of a mutant DNA remain the same, it is sometimes possible to detect changes in sequence by their effects on the migration of short fragments of single-stranded DNA, in a technique called SSCP. By comparing the migration patterns of mutated and non-mutated DNA strand in the non-denaturing gel, the presence or absence of mutation can be determined(see: Mol. Cell Biol., 289, 1995).

In addition to several techniques mentioned above, mutations can be detected by direct DNA sequencing through gel electrophoresis or using instruments(see: Mol. Cell Biol., 245-248, 1995). The said techniques which typically involve the use of radioactive material, fluorescent dye or enzyme for detection of signals, have

drawbacks such as safty problems and requiring high cost in waste disposal in case of radioactive labeling, and if not radioactive labeling, cumbersome and time consuming manipulation due to the use of membranes or performing an acrylamide gel electrophoresis.

Meanwhile, a microwell plate which has been used for identifying a protein with an antibody or identifying a specific nucleotide sequence by hybidizing with DNA or oligonucleotide can be used for determining the presence or absence of a mutation by employing the method of identifying a specific nucleotide sequence. For instance, a sample DNA is affixed to the microwell, and then identified with an oligonucleotide probe corresponding to the normal DNA sequence to be identified: i.e., a sample DNA amplified by PCR technique is affixed to a microwell, hybridizes with a biotin-bound probe, followed by the assessment of hybridization using an enzyme(see: Mol. Cell Probes, 6(1):79-85, 1992). The prior art method is, however, proven to be less satisfactory in the senses that it takes much time to analyze a sample DNA since the sample DNA has to be affixed to the microwell after collecting from a patient; due to the length of DNA, a sample DNA amplified by PCR may have a secondary structure which cause inhibition of hybridization with a probe; long-length DNA molecule may prevent its binding to the well(see: Anal. Biochem., 138-142, 1991); and, since a sample DNA is affixed to a microwell after denaturation into single strands, renaturation of the single-stranded DNA takes place easily during the period of experiment, which lowers the binding ability of sample DNA to the probe.

Summary of the Invention

In accordance with the present invention, it has been discovered that the mutations of DNA sequence can be identified in an economical, simple and safe manner by using microwells and a degradation enzyme which can bind to

biotin.

A primary object of the present invention is, therefore, to provide a method for identifying mutations in a DNA sequence whose corresponding normal sequence has already been determined, by amplifying target(sample) DNA through PCR and using microwells.

The other object of the invention is to provide a kit therefor with convenience.

BRIEF DESCRIPTION OF THE DRAWINGS

The above and the other objects and features of the present invention will become apparent from the following descriptions given in conjunction with the accompanying drawing, in which:

Figure 1 is a graph showing the results of identification of DNA mutations employing the method of the invention.

DETAILED DESCRIPTION OF THE INVENTION

The method for identifying DNA mutation using microwells comprises the steps of: preparing amplified biotinylated DNA fragments of a portion of nucleotide sequence to be identified by PCR using a biotin-bound primer; preparing a probe comprising normal sequence corresponding to the DNA sequence to be identified; affixing the probe thus prepared to the amine group of microwell; adding biotinylated DNA fragments thus prepared to the probe-affixed microwell; adding a streptavidin-linked degradation enzyme to the microwell in order to bind the degradation enzyme to biotin moiety of the probe; and, adding a substrate to be reacted with the degradation enzyme and detecting the color or absorbance change caused by degradation of the substrate.

Also, a kit for identifying DNA mutation comprises a microwell whose inside has amine group, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide(EDC) solution and 1-methylimidazole solution, pH 7.0 for affixing a probe, 0.4M NaOH/0.25% Tween-20 solution for removing unaffixed probe, a solution containing dH_2O, 20xSSPE/0.0167% Triton X-100 and salmon sperm DNA(10mg/ml) for blocking microwell surface, a solution of 0.5xSSC/0.1% Tween-20 for removing unhybridized biotinylated sample DNA fragments, a streptavidin-linked degradation enzyme to be bound to biotin, 100mM Tris-HCl(pH 7.5) solution containing 150mM NaCl for binding of streptavidin to biotin, 100mM Tris-HCl(pH 7.5) solution containing 150mM NaCl/0.1% Tween-20 for removing unbound streptavidin, and a substrate for the streptavidin-linked degradation enzyme.

In describing the specification, the term of 'mutation' is employed to mean a sequence variation such as substitution in which one or more nucleotides are substituted with another nucleotide(s), deletion in which one or more of existing nucleotides are deleted, and insertion in which one or more of additional nucleotides are inserted.

Mutations caused by one or more nucleotide changes frequently occur in microorganisms. For example, Mycobacterium tuberculosis which has a nucleotide change at a specific position is known to be resistant to rifampicin(see: Lancet 341:647-650, 1993). Therefore, detection of the presence or absence of mutations, moreover, identification of changed nucleotide sequences may be very useful in treatment of patients.

In accordance with the present invention, the method for identifying DNA mutation can be applied to detect not only the presence of mutations but also identify the types of mutations such as nucleotide substitution, insertion and deletion with ease. The method involves the following steps of preparing amplified sample DNA, preparing a probe,

affixing the probe to a microwell and detection of mutation by binding of sample DNA to the probe.

Step 1: Amplification of sample DNA

A portion of DNA sequence to be identified for mutation is amplified by polymerase chain reaction(PCR) employing a biotin-bound primer to give amplified biotinylated DNA fragments of sample: Since the PCR is carried out by employing a biotin-bound primer, the amplified product by PCR is a mixture of biotinylated and non-biotinylated nucleotide sequences which are complementary to each other.

Step 2: Preparation of probes

DNA probes which comprise normal sequences corresponding to the DNA sequences to be identified are prepared: The probes thus prepared consist of more than 10 nucleotides and contains a phosphate moiety at 5'end which enables the probe to be affixed to the amine group of microwell.

Step 3: Affixture of probes

The probes thus prepared are affixed to amine group on the inner surface of microwell: Since nucleotide sequences of synthesized probe may be reannealed spontaneously, the single stranded probes can be maintained by heating the probes for 5 to 15 minutes, most preferably for 10 minutes, at 90 to 100℃, most preferably at 94℃, followed by immediate cooling in ice water. Ice cold solutions of 10mM 1-methylimidazole, pH 7.0 and 10 mM 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide(EDC), pH 7.0 which catalyze the covalent-binding reaction of phosphate moiety of oligonucleotide and amine group on microwell are added to the single-stranded DNA probe. The probe

containing mixture was added to the amine-derivatized microwell, and the microwells are sealed with tape to prevent evaporation of reaction mixture, followed by incubation for 5 to 9 hours, most preferably for 7 hours, at 40 to 60℃, most preferably at 50℃. In this way, the probes are covalently affixed on the inner surface of microwell. Probe-affixed microwells are washed with 0.4M NaOH/0.25% Tween-20, and then with distilled water at room temperature.

Step 4: Addition of sample to the microwell

Biotinylated PCR fragments obtained in the previous step are added to the probe-affixed microwells: First, a solution containing dH_2O, 20XSSPE/0.0167% Triton X-100 and salmon sperm DNA(10mg/ml) is added to microwells, and incubated for 10 to 20 minutes, most preferably for 15 minutes, at 40 to 60℃, most preferably at 50℃, to prevent biotinylated sample from binding to free amine group on the microwell surface. In order to denature biotinylated double-stranded DNA fragments obtained in Step 1 to allow hybridization with the probe, they are heated for 5 to 15 minutes, most preferably for 10 minutes, at 90 to 98℃, most preferably at 94℃, and chilled immediately in ice water. And then, a solution containing dH_2O, 20XSSPE/0.0167% Triton X-100 and salmon sperm DNA (10mg/ml) is added to the single-stranded DNA sample. The mixture is introduced into the microwells, incubated for 5 to 15 hours, most preferably for 10 hours, at 50 to 70℃, most preferably at 60℃. After incubation, the residual mixture is removed, and microwells are washed with 0.5xSSC, 0.1% Tween-20. The sample with changed nucleotide sequence(s) would show low affinity for the probe affixed to microwell while the sample with normal sequence would show high affinity. Accordingly, biotin of the sample DNA fragments can be captured to the microwell, and the affinity difference between mutated and non-mutated sequences for

the probe can be measured in terms of the difference in density of microwell-bound biotin.

Step 5: Addition of a degradation enzyme

Streptavidin-linked degradation enzyme is added to the microwell in order to bind the degradation enzyme to biotin moiety of the probe-captured sample DNA fragment: To detect the difference in biotin density bound to the microwell of mutated and non-mutated DNA samples, degradation enzyme is bound to biotin. The degradation enzyme hydrolyzes a chromogenic substrate, and resulting changes in color intensity or absorbance can be measured. For example, streptavidin-alkaline phosphatase can be used; microwells are washed with a buffer solution of 100mM Tris-HCl(pH 7.5) containing 150mM NaCl/0.1% Tween-20, and streptavidin-akaline phosphatase diluted with a buffer solution of 100mM Tris-HCl(pH 7.5) containing 150mM NaCl, is added to the microwell, and incubated for 30 to 90 minutes, most preferably for 60 minutes, at 35 to 45℃, most preferably at 40℃. After removal of the residual reaction mixture, a buffer solution of 100mM Tris-HCl(pH 7.5) containing 150mM NaCl/0.1% Tween-20 is added, incubated for 5 to 15 minutes, most preferably for 10 minutes, at 50 to 70℃, most preferably at 60℃, and then washed.

Step 6: Detection of enzyme reaction

The substrate for streptavidin-linked degradation enzyme was added to the microwell, and color or absorbance changes by enzyme reaction are detected: Synthetic peptides which show color or absorbance changes are desirable as substrates in degradation by the enzyme-substrate reaction. For example, in case of using streptavidin-alkaline phosphatase as a degradation enzyme, 100㎕ of pNPP(p-nitrophenyl phosphate) is added to the microwell, and

incubated for 60 to 120 minutes, most preferably for 90 minutes, at room temperature. After the reaction is stopped by addition of 1M NaOH, absorbance is measured at 405nm. Although the conventional spectrophotometer can be used to measure the absorbance, ELISA reader is a more desirable instrument to measure the absorbance of enzyme-substrate reaction mixture in microwells.

Also, the present invention provides a kit comprising the following components to implement the method described above: microwell whose inside has amine group, EDC solution and 1-methylimidazole solution, pH 7.0 for affixing a probe, 0.4M NaOH/0.25% Tween-20 solution for removing unaffixed probe, a solution containing dH_2O, 20xSSPE/0.0167% Triton X-100 and salmon sperm DNA(10mg/ml) for blocking free amine groups on microwell surface, a solution of 0.5xSSC/0.1% Tween-20 for removing unhybridized biotinylated sample DNA fragments, a streptavidin-linked degradation enzyme to be bound to biotin, 100mM Tris-HCl(pH 7.5) solution containing 150mM NaCl for binding of streptavidin to biotin, 100mM Tris-HCl(pH 7.5) solution containing 150mM NaCl/0.1% Tween-20 for removing unbound streptavidin, and a substrate for the streptavidin-linked degradation enzyme.

In accordance with the present invention, mutated and non-mutated DNA samples can be differentiated by measuring biotin density bound to the microwell. That is, a degradation enzyme is bound to biotin, and activity of the bound enzyme can be assayed by addition of a substrate reacting with the degradation enzyme. In this way, the presence of mutation in the sample DNA can be detected and, furthermore, the type of the mutation can be identified. Therefore, in any experiments to detect DNA mutations, the results can be obtained in a more economical, simpler and safer way than any other conventional method as long as the portion of DNA to be identified can be amplified and a probe comprising the normal sequence corresponding to the

DNA to be identified can be prepared.

The method for identifying DNA mutation using microwells of the present invention provides the following advantages over conventional methods:

1. Since the same results can be obtained by using smaller amount of oligonucleotides than conventional methods, the invented method is more economical. Similar results in terms of absorbance can be obtained with 100ng of probe/well by employing the method of the invention while prior art method requires 600ng/well(see: Mol. Cell. Probes, 12:407-416, 1998).

2. In the prior art, microwells pretreated for binding oligonucleotides later were provided. Instead, oligonucleotide-affixed microwells are provided in the present invention, which in turn reduces the costs for oligonucleotide synthesis and microwell preparation as well.

3. When mutation is detected by using a conventional microwell having amine group to which oligonucleotides are to be bound, high absorbance of negative control is a major problem. By using microwells of present invention, the absobance of negtive control can be reduced remarkably.

4. Conventional methods for detecting DNA mutation in which sample DNA is affixed to microwell have problems such as secondary structures caused by length of sample DNA, weak binding affinity and complementary binding between sample nucleotide strands. These problems are solved in the invention by preaffixing DNA probe to the microwell.

5. The prior art uses membranes or gels whose handling is very cumbersome, and requires many complicated work accompanying the difficulties in handling large number of samples, while the invented method guarantees simplicity by performing entire steps in one well.

6. The prior art has many variations such as different ways of collecting samples and conducting experiments, which is an obstacle to performing experiments in a reproducible manner. The kit of the invention not

only provides the consistency in experimental condition, but also can be stored for a long period of time, which enables detection of large number of samples at the same time. Especially, since experimental errors may be avoided by using instruments, experimental data or type of samples can be analyzed statistically.

7. Results can be identified visually. That is, color changes resulting from enzymatic reaction can be detected with naked eyes, therefore, due to the obviousness of the result, the reseacher can have confidence in the results.

The present invention is further illustrated in the following examples, which should not be taken to limit the scope of the invention.

Example 1: Identification of DNA mutation using microwells

The method for identifying DNA mutation of the invention involves procedures to detect the presence of mutation in patient's DNA by affixing a probe comprising the normal sequence corresponding to the DNA to be identified to the microwell, and then by assessing stability of the hybrid between a DNA sample and a complementary oligonucleotide probe immobilized on the microwell. As a representative example, the present inventors employed a direct sequenced region which is known to be uniquely found in genome of a Mycobacterium tuberculosis, BCG(Calmette-Guerin bacillus), and assessed the stability of duplexes of a sample DNA of a patient and various probes which include a probe of the normal nucleotide sequence of direct sequenced region, probes containing different types of mutations in the direct sequenced region, and a probe which is not related to the direct sequence as a negative control.

Example 1-1: The use of a probe comprising normal

nucleotide sequence

First, primer a comprising a biotin-bound nucleotide sequence of 5'-GGTTTTGGGTCTGACGAC-3' (SEQ ID: 1) and primer b of 5'-CCGACAGGGGACGGAAAC-3' (SEQ ID: 2) were synthesized by the conventional method. And then, PCR was performed in a PCR mixture containing DNA(comprising direct sequence of BCG) to a final concentration of 200 ng/㎕, 0.5㎕ of primer a(100pmol/㎕), 0.5㎕ of primer b(100pmol/㎕), 0.4㎕ of dNTP(25mM), 10xTaq buffer, 3㎕ of $MgCl_2$(25mM), 0.2㎕ of Taq(5unit/㎕, Promega, U.S.A.) and 40.4㎕ of dH_2O, with 30 cycles of denaturation(for 1min. at 94℃), annealing(for 1min. 30sec. at 55℃) and extension(for 1min. 30sec. at 72℃).

Subsequently, probe #1 comprising a normal nucleotide sequence of 5'-TTGACCTCGCCAGGAGAGAAGATCA-3' (SEQ ID: 3) and probe #2 comprising a normal nucleotide sequence of 5'-TCCGTACGCTCGAAACGCTTCCAAC-3') was synthesized. 10 pmol aliquots of each probe were heated for 10 minutes at 94℃, cooled down for 10 minutes in ice water, condensed by centrifugation. And then, a solution of EDC and 1-methylimidazole pH 7.0 was added to the each aliquot of the probe to a final concentration of 10mM. 100㎕ of each probe mixture thus prepared was introduced into microwells(Nunc, Denmark) on ice, incubated for 7 hours at 50℃. After removing the residual probe mixture, a solution containing 138㎕ of dH_2O, 20xSSPE/0.0167% Triton X-100, and 2㎕ of salmon sperm DNA(10mg/ml) was added to the microwells, and incubated for 20 minutes at 50℃ for blocking free amine group on the microwell surface.

After removing the blocking solution, a sample mixture containing 68㎕ of dH_2O, 30㎕ of 20xSSPE/0.0167% Triton X-100, 1㎕ of salmon sperm DNA(10mg/ml) and 1㎕ of PCR amplified sample DNA prepared above was added to each microwell, and incubated for about 10 hours at 60℃. After removing the mixture, each microwell was washed 3 times with 200㎕ of 0.5x SSC, 0.1% Tween-20, and the same

solution was added to incubate for 15 minutes at 60℃. After washing 3 times with the same solution, 100 ㎕ of streptavidin-alkaline phosphatase prepared by 3000-fold dilution with a buffer solution of 100mM Tris-HCl(pH 7.5) containing 150mM NaCl was added to the microwell, and incubated for 1 hour at 40℃.

Subsequently, the microwells were washed 3 times with a buffer solution of 100mM Tris-HCl (pH 7.5) containing 150mM NaCl/0.1% Tween-20, and subjected to 3 times of incubation for 5 minutes at 60℃ and washing with the same solution. 100 ㎕ of pNPP(N7653, Sigma, U.S.A) was added to each well, incubated for 90 minutes, and then the absorbance was measured at 405nm(see: Figure 1).

Example 1-2: The use of a probe with a substituted nucleotide

To identify a substitution mutation, probe #11 of 5'-TTGACCTCGCCAGAAGAGAAGATCA-3'(SEQ ID: 5) was synthesized by substituting the 14th nucleotide of probe #1, G with A, and probe #21 of 5'-TCCGTACGCTCGAGACGCTTCCAAC-3'(SEQ ID: 6) was synthesized by substituting the 14th nucleotide of probe #2, A with G. Then, identification of DNA mutation was performed in an analogous manner as in Example 1-1, except for employing the use of probes #11 and #21 instead of probes #1 and #2(see: Figure 1).

Example 1-3: The use of a probe with an inserted nucleotide

To identify an insertion mutation, probe #12 of 5'-TTGACCTCGCCAGTGAGAGAAGATCA-3'(SEQ ID: 7) was synthesized by inserting T between 13th and 14th nucleotide of probe #1. Then, identification of DNA mutation was performed in an analogous manner as in Example 1-1, except for employing the use of probe #12 instead of probes #1 and #2(see: Figure 1).

Example 1-4: The use of a probe with a deleted nucleotide

To identify a deletion mutation, probe #13 of 5'-TTGACCTCGCCAGAGAGAAGATCA-3'(SEQ ID: 8) was synthesized by deleting 14th nucleotide, G of probe #1. Then, identification of DNA mutation was performed in an analogous manner as in Example 1-1, except for employing the use of probe #13 instead of probes #1 and #2(see: Figure 1).

Example 1-5: The use of a negative control probe

For a negative control, probe #C of 5'-GGAGCTTTCCGGCTTCTATCAGGTA-3'(SEQ ID: 9) comprising a nucleotide sequence which is found uniquely in a Mycobacterium tuberculosis, H37Rv, was synthesized. Then, identification of DNA mutation was performed in an analogous manner as in Example 1-1, except for employing the use of probe #C(see: Figure 1).

Figure 1 shows a graph, based on a direct sequenced region which is known to be uniquely found in the genome of Mycobacterium tuberculosis, BCG, demonstrating the identification of DNA mutation by assessing the stability of the duplex of a sample DNA from a patient and various probes which include a probe comprising a normal nucleotide sequence of direct sequenced region, probes containing different types of mutations in the direct sequenced region, and a probe which is not related to the direct sequence as a negative control. In Figure 1, No DNA represents a control without probe; No.1, a negative control, probe #C; No.2, probe #1 of normal sequence; No.3, probe #11 of substitution mutant; No.4, probe #13 of deletion mutant; No.5, probe #12 of insertion mutant; No.6, probe #2 of normal sequence; and, No.7, probe #21 of substitution mutant, respectively.

As shown in Figure 1, since the OD value from the

hybridization of the sample DNA with the probe of normal sequence showed the highest value, the sample DNA from a patient turned out to comprise the normal sequence. On the other hand, although the hybridization of a sample DNA with probes of mutated sequences made differences in OD values for each mutated probe more or less, the values were significantly lower than that for a probe of normal sequence, thus assuring that the presence of mutation can be clearly identified by the method of the invention.

The application of the invention make it possible to identify the position and the type of mutation by employing probes comprising each mutated sequence with one or more nucleotide changes.

As clearly illustrated and demonstrated above, the present invention can be applied to identify not only the presence of one or more mutations but also the types of mutations such as substitution, deletion and insertion in a more economical, simpler and safer way than any other conventional method.